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ACCESSORY GROWTH FACTOR REQUIREMENTS OF THE MEMBERS OF THE GENUS PASTEURELLA

A PART OF A DISSERTATION SUBMITTED TO THE FACULTY OF THE DIVISION OF THE BIOLOGICAL SCIENCES IN CANDIDACY FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF BACTERIOLOGY AND PARASITOLOGY
1942

By
SAM BERKMAN

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SAM BERKMAN

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INTRODUCTION

Since preliminary work in this laboratory has shown that most of the members of the Pasteurella group could not be cultivated in "synthetic" mediums composed of amino acids, inorganic salts and glucose, a study of the accessory growth factor requirements of these organisms was undertaken. The organisms commonly included in this group are: (1) the hemorrhagic septicemia strains, which at present are designated according to their zoological source, such as Pasteurella avicida, Pasteurella boviseptica, etc., (2) Pasteurella pestis, (3) Pasteurella pseudotuberculosis and (4) Pasteurella tularense. Pasteurella hemolytica is the name suggested by Newsom and Cross[1] for those organisms isolated from cases of hemorrhagic septicemia in animals, which nevertheless show certain physiological characteristics different from the typical forms (Past. avicida, Past. boviseptica, etc.). There has been some discussion as to whether or not the causative agent of tularemia belongs in the genus Pasteurella.

A report of the requirements of the first sub-group, the hemorrhagic septicemia organisms, has been made earlier.[2] In the present investigation the study of these organisms has been extended and the other representatives of the group have been included.

During the course of this work 17 strains of the hemorrhagic septicemia pasteurellas, 15 strains of Past. tularense, 5 strains of Past. pestis, 5 strains of Past. pseudotuberculosis and 1 strain of Past. hemolytica were studied. With the exception of the tularemia strains the cultures used were, for the most part, older laboratory cultures received from different parts of the United States. Most of the tularemia strains had been isolated only a few weeks before use in this study.

METHODS

Basal mediums.—A medium prepared from hydrolyzed purified gelatin was used for most of the work. It had the following composition:

Basal Medium A

Hydrolyzed purified gelatin	5.0 gm.
Sodium chloride	5.0 gm.
Dipotassium hydrogen phosphate	2.0 gm.
Magnesium sulfate	0.05 gm.
Calcium chloride	0.01 gm.
Hoagland salt mixture	1.0 cc.
dl-valine	0.02 gm.
dl-serine	0.01 gm.
1(-)-tyrosine	0.02 gm.
1(-)-tryptophane	0.02 gm.
1(-)-cystine	0.02 gm.
dl-methionine	0.02 gm.
1(-)-histidine HCl	0.02 gm.
Threonine	0.01 gm.
Glucose	3.0 gm.
Redistilled water	1,000.0 cc.

The p_H is adjusted to 6.8–7.0 with normal

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The author wishes to express his indebtedness to Dr. Stewart A. Koser for much helpful advice and criticism during the course of this investigation. Thanks are due also to Dr. Albert Dorfman for valuable suggestions and for several of the compounds used in this study.

1. J. Am. Vet. M. A. **33:** 711, 1932.

2. Berkman, S., Saunders, F., and Koser, S. A.: Proc. Soc. Exper. Biol. & Med. **44:** 68, 1940.

sodium hydroxide. The medium is then tubed in 5 cc. quantities and sterilized in the autoclave.

This medium was deficient in certain substances needed by some members of this group of organisms, for many of them failed to develop in the basal medium alone but did develop on the addition of a mixture of known accessory growth substances.

In later work a synthetic medium was used also. The composition of this medium was as follows:

Basal Medium B

Sodium chloride	5.0 gm.
Calcium chloride	0.01 gm.
Disodium hydrogen phosphate	1.0 gm.
Magnesium sulfate	0.1 gm.
Glutamic acid	0.5 gm.
Alpha alanine	0.5 gm.
Glycine	0.2 gm.
1(+)-lysine 2 HCl	0.2 gm.
1(−)-tryptophane	0.2 gm.
1(−)-histidine 2 HCl	0.2 gm.
1(−)-cystine	0.15 gm.
dl-valine	0.1 gm.
1(−)-leucine	0.1 gm.
dl-phenyl alanine	0.1 gm.
1(−)-proline	0.1 gm.
1(−)-hydroxyproline	0.1 gm.
dl-methionine	0.1 gm.
1(+)-arginine HCl	0.1 gm.
1(−)-tyrosine	0.05 gm.
dl-aspartic acid	0.02 gm.
dl-serine	0.02 gm.
Threonine	0.02 gm.
Glucose	3.0 gm.
Redistilled water	1000.0 cc.

The p_H is adjusted to 6.8–7.0 with normal sodium hydroxide. The medium is then tubed in 5 cc. quantities and sterilized in the autoclave.

The amino acid requirements of the organisms in this group are not known, but the combination above supplies the usual amino acids. Also, little is known of the exact needs of these organisms as concerns inorganic salts and certain physical requirements of the medium. In some experiments a mixture of some of the rarer salts (Hoagland's salt mixture) was added to the synthetic medium, but the addition of these salts did not have any beneficial effect on growth.

Accessory growth substances.—The accessory growth substances were sterilized by filtration through sintered glass filters and added aseptically to the basal medium. The accessory mixture used in the first part of the work consisted of nicotinamide, thiamin, cocarboxylase, riboflavin, pantothenic acid, nicotinamide methiodide (0.1 μg. each per cc.), diphosphopyridine nucleotide, beta-alanine, sodium pyrophosphate (0.5 μg. each per cc.), pyridoxine (0.4 μg. per cc.), inositol (5.0 μg. per cc.) and glutamine (15.0 μg. per cc.).

Certain other compounds and also some derivatives of the known accessories were used in the latter part of the investigation. These will be mentioned in the appropriate sections.

Method of inoculation.—For most of the experiments the test organisms were grown on meat infusion agar for 24 hours at 37 C. A very light suspension of each organism was made by removing a small amount of growth from the slant on the tip of a straight inoculating needle and suspending it in a tube containing 5 cc. of phosphate-buffered salt solution. After thoroughly mixing the material, 0.1 cc. of this suspension was added to each 5 cc. of medium.

Plate counts of the inoculum made at various times showed that, as a rule, 10,000 to 20,000 organisms were inoculated into each cc. of basal medium. In a few experiments further dilutions of the inoculum, one-tenth and one-hundredth of this amount, were used.

EXPERIMENTAL RESULTS

Table 1 shows the growth of representative members of the Pasteurella group in the basal medium of hydrolyzed gelatin and in the same medium plus the mixture of accessory substances. Identical results were secured on repeated tests. This table indicates that there is some correlation between subdivisions based on the conventional methods of classification and the accessory growth factor requirements of the members of the group. The typical hemorrhagic septicemia organisms of animals cannot grow in the basal medium of hydrolyzed gelatin but for the most part can grow in this medium when a mixture of known accessory substances is added. The tularemia organisms fail to develop in the presence of the accessories unless a larger inoculum is used and even then the growth is slow. The plague bacillus, Past. pseudotuberculosis, and Past. hemolytica de-

TABLE 1.—*Growth of Members of the Pasteurella Genus in the Hydrolyzed Gelatin Basal Medium and with Addition of Accessory Substances*

Pasteurella	Basal Medium				Basal Medium plus Accessory Mixture			
	days*							
	1	2	4	7	1	2	4	7
equiseptica S230	0	0	0	0	++	+++	+++	+++
cuniculicida 7229	0	0	0	0	0	0	±	+
oviseptica 1	0	0	0	0	0	++	++	++
oviseptica 2	0	0	0	0	++	+++	+++	+++
bubaliseptica 41	0	0	0	0	+++	+++	+++	+++
bubaliseptica 888	0	0	0	0	+++	+++	+++	+++
bubaliseptica MH718	0	0	0	0	+++	+++	+++	+++
boviseptica 53	0	0	0	0	+++	+++	+++	+++
boviseptica 221	0	0	0	0	+++	+++	+++	+++
boviseptica 590L	0	0	0	0	+++	+++	+++	+++
boviseptica 18	0	0	0	0	±	+	++	++
suiseptica 100	0	0	0	0	+++	+++	+++	+++
suiseptica DM2	0	0	0	0	+++	+++	+++	+++
avicida 1525	0	0	0	0	+++	+++	+++	+++
avicida NY81	0	0	0	0	+++	+++	+++	+++
avicida	0	0	0	0	±	++	++	++
aviseptica 351	0	0	0	0	+++	+++	+++	+++
hemolytica	+++	+++	+++	+++	+++	+++	+++	+++
pestis 122	0	0	+	+	0	0	+	+
pestis 125	+++	+++	+++	+++	+++	+++	+++	+++
pestis 6386	0	++	+++	+++	0	++	+++	+++
pestis 6387	0	+++	+++	+++	0	+++	+++	+++
pestis 6388	0	++	+++	+++	0	++	+++	+++
pseudotuberculosis 907	+++	+++	+++	+++	+++	+++	+++	+++
pseudotuberculosis 908	+++	+++	+++	+++	+++	+++	+++	+++
pseudotuberculosis 6903	+++	+++	+++	+++	+++	+++	+++	+++
pseudotuberculosis 6902	+++	+++	+++	+++	+++	+++	+++	+++
kutscheri 6944	+++	+++	+++	+++	+++	+++	+++	+++
tularense 395	0	0	0	0	0	0	++	+++
tularense 399	0	0	0	0	0	0	++	+++
tularense 405	0	0	0	0	0	0	++	+++
tularense 408	0	0	0	0	0	0	++	++
tularense 410	0	0	0	0	0	0	++	+++
tularense 413	0	0	0	0	0	0	++	+++
tularense 421	0	0	0	0	0	0	++	++
tularense 424	0	0	0	0	0	0	++	++
tularense 425	0	0	0	0	0	0	+++	+++
tularense 426	0	0	0	0	0	0	+++	+++
tularense Tennessee	0	0	0	0	0	0	++	+++
tularense 407	0	0	0	0	0	0	++	+++
tularense 396	0	0	0	0	0	0	++	+++
tularense Pack	0	0	0	0	0	0	++	+++
tularense 38	0	0	0	0	0	0	0	0

0 =no visible growth, ± =very light turbidity just at point of visibility, + to +++ =light to pronounced turbidity.
* All cultures were held for at least 10 days at 37 C. Usually there was no change other than that shown.
The Past. tularense strains were not able to develop in the presence of the accessory mixture with the light inoculum used for the other members of the group, but they did develop, as shown above, when the inoculum was increased somewhat.

velop readily in the basal medium without the addition of any accessory substances. In subsequent work each of these groups was studied in more detail and the results for each will be presented separately.

HEMORRHAGIC SEPTICEMIA PASTEURELLAS

Since the typical hemorrhagic septicemia strains from animal infections developed in the presence of the mixture of accessory growth factors (table 1), the first problem undertaken was to determine more specifically the needs of these strains. In order to determine which of these factors was essential for growth the original mixture was progressively simplified. It was found that the accessory factors in the mixture required for growth in the hydrolyzed gelatin basal medium were nicotinamide (or diphosphopyridine nucleotide) and pantothenic acid. Neither alone supported growth. Thirteen of the 17 strains studied grew readily in the presence of nicotinamide and pantothenic acid. These results were verified in repeated tests and are presented in table 2. In order to be certain that growth of these organisms was not due to materials which might have been carried over

TABLE 2.—*Effect of Addition of Nicotinamide and Pantothenic Acid upon Growth of 17 Strains of Hemorrhagic Septicemia Pasteurellas*

Pasteurella	Nicotinamide*		Pantothenic Acid*		Nicotinamide plus Pantothenic Acid	
	days					
	1	2	1	2	1	2
equiseptica S230	0	0	0	0	++	+++
cuniculicida 7229	0	0	0	0	0	±
oviseptica 1	0	0	0	0	+	++
oviseptica 2	0	0	0	0	++	++
bubaliseptica 41	0	0	0	0	+++	+++
bubaliseptica MH718	0	0	0	0	++	+++
bubaliseptica 888	0	0	0	0	+++	+++
boviseptica 53	0	0	0	0	+++	+++
boviseptica 221	0	0	0	0	++	+++
boviseptica 590L	0	0	0	0	+++	+++
boviseptica 18	0	0	0	0	+	+
suiseptica 100	0	0	0	0	++	+++
suiseptica DM2	0	0	0	0	+++	+++
avicida 1525	0	0	0	0	+++	+++
avicida NY81	0	0	0	0	+++	+++
avicida	0	0	0	0	+	++
aviseptica 351	0	0	0	0	+++	+++

* Both nicotinamide and pantothenic acid were added in amounts of 0.1μg. per cc. of basal medium. All cultures were held for 10 days at 37 C. The results were essentially the same as those shown for 2 days.

with the original inoculum, successive transplants in the same medium were made with a number of characteristic strains by inoculating a loopful of the culture to another tube of the same basal medium plus the 2 accessories. The organisms grew readily in 10 successive transplants by this method.

The other 4 strains generally gave a scantier though still distinct growth, indicating that other factors or conditions were needed for ready cell multiplication by these strains. The further addition of para-amino-benzoic acid, vitamin K, choline, oleic acid and crystalline biotin did not stimulate growth of the latter strains. Addition of a biotin concentrate, however, induced more vigorous growth of 3 of these 4 cultures. One sample of the biotin concentrate was kindly supplied by Dr. W. H. Peterson of the University of Wisconsin and a second sample by General Biochemicals, Inc. The stimulating effect of the biotin concentrate is apparently due to some substance, other than biotin, which is present as an impurity in the concentrate, or to this impurity plus the biotin. Table 3 shows the growth promoting effect of the biotin concentrate for the 3 strains that were stimulated by it.

The pantothenic acid used was obtained as the calcium salt. It appeared improbable that the stimulative effect of calcium pantothenate was due to calcium since calcium chloride was included in the basal medium. Addition of calcium chloride to give an amount of calcium equivalent to that in calcium pantothenate did not promote growth or metabolic activity.

TABLE 3.—*Effect of Addition of Biotin Concentrate on Growth of Some Fastidious Strains of Hemorrhagic Septicemia Pasteurellas*

Basal Medium A with	Amount added (μg. per cc. medium)	Pasteurella					
		oviseptica 1		boviseptica 18		avicida	
		days					
		1	2	1	2	1	2
Nicotinamide Pantothenic acid	0.1 0.1	+	+	+	+	+	+
Nicotinamide Pantothenic acid Biotin concentrate	0.1 0.1 0.15	++	++	++	++	+++	+++
Control		0	0	0	0	0	0

End-point experiments.—In order to determine what quantities of nicotinamide and pantothenic acid are needed by the typical hemorrhagic septicemia pasteurellas, decreasing amounts of each of the compounds were used in the presence of an excess of the other. The results of these tests are shown in table 4.

TABLE 4.—*End-point Experiments with Nicotinamide and Pantothenic Acid*

Basal Medium A and	Amount added (μg. per cc. Medium)	Pasteurella avicida 1525		Pasteurella suiseptica DM2		Pasteurella bubaliseptica MH718	
		days 1	2	1	2	1	2
Nicotinamide (0.1μg. per cc.) plus							
Pantothenic acid	0.02	+++	+++	+++	+++	+++	+++
	0.01	+++	+++	+++	+++	+++	+++
	0.004	+	+	++	++	++	++
	0.002	±	±	±	±	+	+
	0.001	0	0	0	0	0	0
Pantothenic acid (0.1 μg. per cc.) plus							
Nicotinamide	0.004	+++	+++	+++	+++	+++	+++
	0.002	+++	+++	+++	+++	+++	+++
	0.001	++	++	++	++	++	++
	0.0004	+	+	±	±	±	±
	0.0002	±	±	0	0	0	0

The end point of pantothenic acid activity, in the presence of an excess of nicotinamide, is about 0.002 μg. per cc. (1×10^{-8}M), and that of nicotinamide, in the presence of an excess of pantothenic acid, is about 0.0004 μg. per cc. (3×10^{-9}M).

Comparative activity of related compounds.—Other workers have shown that compounds closely related chemically to an active factor can substitute, at times, for the natural compound. Among the compounds tested in this study were the component parts of pantothenic acid, a number of pyridine derivatives, several thiazole and pyrazine compounds and some miscellaneous compounds.

The structure of pantothenic acid has been determined by Stiller, Harris et al.[3] who found it consisted of beta-alanine and β, β-dimethyl-α-hydroxy-γ-butyrolactone attached through a peptide linkage. The component parts of the pantothenic acid molecule were tested to see if they would replace the complete molecule, but it was found that they would not replace it either separately or when supplied together. Thus these organisms apparently are not able to combine the component parts of the molecule. These results are presented in table 5 for 3 of the 16 organisms tested. The findings for the others were the same. Two samples of each of the compounds, obtained from different sources,* were tested. The results were uniformly negative in several different tests.

A study was made of the effect of a number of compounds when substituted for nicotinamide. The results of these tests are shown in table 6. Of the substances tested only di- and triphosphopyridine nucleotide were able to substitute for nicotinamide. This would be expected since nicotinamide is a constituent of these two substances. The others failed to substitute for nicotinamide even though some of them are rather closely related to it and have been shown to replace it, to some extent at least, in the case of other organisms.

3. J. Am. Chem. Soc. 62: 1785, 1940.

* Merck and Company and the National Oil Products Company kindly supplied the samples of β,β-dimethyl-α-hydroxy-γ-butyrolactone.

TABLE 5.—*Effect of Replacing Pantothenic Acid with Its Component Parts*

Basal Medium A and Nicotinamide plus	Amount added (μg. per cc.)	Pasteurella avicida		Pasteurella boviseptica 1		Pasteurella bubaliseptica	
		days 1	2	1	2	1	2
Pantothenic acid	0.1	+++	+++	+++	+++	+++	+++
Beta-alanine	0.1	0	0	0	0	0	0
Butyrolactone*	0.1	0	0	0	0	0	0
Beta-alanine plus butyrolactone	0.1 each	0	0	0	0	0	0
Nothing (control)	0	0	0	0	0	0	0

* β, β-dimethyl-α-hydroxy-γ-butyrolactone.

TABLE 6.—*Effect of Substituting Various Compounds for Nicotinamide*

Basal Medium A and Pantothenic Acid plus	Amount added	Pasteurella avicida 1525		Pasteurella boviseptica 530		Pasteurella suiseptica 100	
		days* 1	2	1	2	1	2
Nicotinamide	1×10^{-6}M	+++	+++	+++	+++	+++	+++
Nicotinic acid	1×10^{-3}M to 1×10^{-6}M	0	0	0	0	0	0
Diphosphopyridine nucleotide	2×10^{-5}M	+++	+++	+++	+++	+++	+++
Triphosphopyridine nucleotide	2×10^{-6}M	+++	+++	+++	+++	+++	+++
Nicotinuric acid	2×10^{-5}M	0	0	0	0	0	0
Nicotinic acid-N-methyl amide	1×10^{-4}M	0	0	0	0	0	0
Nicotinamide methiodide	1×10^{-4}M	0	0	0	0	0	0
Methyl nicotinate	8×10^{-6}M	0	0	0	0	0	0
Ethyl nicotinate	7×10^{-6}M	0	0	0	0	0	0
Propyl nicotinate	6×10^{-6}M	0	0	0	0	0	0
Butyl nicotinate	6×10^{-6}M	0	0	0	0	0	0
Coramine	1×10^{-4}M	0	0	0	0	0	0
Trigonelline	1×10^{-4}M	0	0	0	0	0	0
2-amino nicotinic acid	3×10^{-4}M	0	0	0	0	0	0
Quinolinic acid	1×10^{-4}M	0	0	0	0	0	0
Dimethyl quinolinate	3×10^{-4}M	0	0	0	0	0	0
Quinolinic imide	3×10^{-4}M	0	0	0	0	0	0
Quinolinic diamide	3×10^{-4}M	0	0	0	0	0	0
Pyridine betaine carboxylic acid	3×10^{-4}M	0	0	0	0	0	0
Pyrazine monocarboxylic acid	1×10^{-4}M	0	0	0	0	0	0
Pyrazine-2-3 dicarboxylic acid	1×10^{-4}M	0	0	0	0	0	0
Thiazole-5-carboxylic acid	1×10^{-4}M	0	0	0	0	0	0
Thiazole-5-carboxylic amide	1×10^{-4}M	0	0	0	0	0	0

* All cultures were held for 10 days at 37 C. Usually there was no change other than that shown.

It will be noted that methyl, ethyl, propyl and butyl nicotinate, nicotinuric acid, nicotinic acid-N-methyl amide, coramine and quinolinic acid all failed to support growth of the hemorrhagic septicemia pasteurellas. These results are in contrast to the findings with respect to other organisms where one or more of these compounds have supported growth of either Staphylococcus aureus,[4] dysentery bacilli[5] or Proteus.[6]

It is especially interesting to note that nicotinic acid when supplied in amounts of approximately 100 to 0.1 μg. per cc. of medium could not replace nicotinamide as a growth factor for these organisms. This is the first instance in which this has been found, for in all the other cases reported for microorganisms and higher forms either the amide or the acid is active. The results were negative in repeated experiments with nicotinic acid from 2 different sources. These same samples of nicotinic acid were active as shown by control tests with dysentery bacilli and Proteus which

4. Knight, B. C. J. G., and McIlwain, H.: Biochem. J. **32:** 1241, 1938; Landy, M.: Proc. Soc. Exper. Biol. & Med. **38:** 504, 1938.

5. Dorfman, A., Koser, S. A., Reames, H. R., Swingle, K. F., and Saunders, F.: J. Infec. Dis. **65:** 163, 1939; Koser, S. A., Dorfman, A., and Saunders, F.: Proc. Soc. Exper. Biol. & Med. **43:** 391, 1940.

6. Lwoff, A., and Querido, A.: Compt. rend. Soc. Biol. **130:** 1569, 1939; Pelczar, M. J., Jr., and Porter, J. R.: J. Bact. **39:** 429, 1940.

require this compound for growth. When nicotinamide was added to the tubes to which nicotinic acid had been added previously and in which growth had not occurred, the hemorrhagic septicemia organisms developed promptly.

The fact that nicotinic acid was not utilized by these organisms may explain their inability to use certain other closely related pyridine compounds. A number of them, especially methyl and ethyl nicotinate, nicotinuric acid and quinolinic acid on hydrolysis would yield nicotinic acid and since the latter compound is not effective it might be expected that these compounds would be inactive.

Since it had been found that diphosphopyridine nucleotide and nicotinamide are active and that nicotinic acid is inactive in stimulating growth of the hemorrhagic septicemia pasteurellas, it was decided to study the effect of these compounds more closely by means of the Thunberg and Warburg technics.

A typical strain of Past. suiseptica was grown in the hydrolyzed gelatin basal medium in the presence of an excess of calcium pantothenate but with a suboptimum amount (0.004 μg. per cc.) of nicotinamide. The cells were collected by centrifugation and were washed with M/20 phosphate buffer at *p*H 7.4, recentrifuged and then resuspended in the phosphate buffer.

It was found that the addition of either diphosphopyridine nucleotide or nicotinamide markedly stimulated both methylene blue reduction and oxygen uptake by the cells grown in this medium deficient in nicotinamide. Nicotinic acid on the other hand, produced no stimulation at all. Similar results were secured on repeated tests. Details of these experiments have been published elsewhere.[7] These results confirmed the growth tests in showing that the hemorrhagic septicemia pasteurellas of animals, though able to utilize nicotinanamide, are apparently unable to make use of nicotinic acid. In contrast to these pasteurellas, organisms such as dysentery bacilli and Proteus show a stimulation of respiration by either nicotinic acid or nicotinamide when grown in the presence or suboptimum amounts of nicotinamide.[8]

Production of diphosphopyridine nucleotide.—It has been frequently suggested that nicotinamide is utilized by cells to synthesize di- or triphosphopyridine nucleotide which function as coenzymes in bacterial respiration. An attempt was made to discover whether or not the organisms studied were able to produce these coenzymes from nicotinamide.

It has been shown that Hemophilus influenzae and Hemophilus parainfluenzae require di- or triphosphopyridine nucleotide (or perhaps a compound similar to them) for growth and that nicotinamide will not replace these coenzymes. To test for the presence of these coenzymes in Pasteurella cultures, 2 day old broth cultures of Past. suiseptica were filtered and the filtrates tested to see if they would support growth of H. parainfluenzae. In some cases the cultures were treated with 0.5 normal hydrochloric acid and heated at 90 C. for 10 minutes before filtration, so as to facilitate extraction of the coenzyme from the cells. There is evidence that this treatment does not break down the coenzymes. Other cultures of Past. suiseptica were filtered without being subjected to acid or heat treatment. All the filtrates were tested for sterility.

It was found that these filtrates were able to induce growth of H. parainfluenzae in a medium in which the latter

7. Koser, S. A., Berkman, S., and Dorfman, A.: Proc. Soc. Exper. Biol. & Med. **47**: 504, 1941.

8. Saunders, F., Dorfman, A., and Koser, S. A.: J. Biol. Chem. **138**: 69, 1941.

organism could otherwise not grow. A typical result is shown in table 7. Evidently the Pasteurella organisms synthesize one or both of the coenzymes or a compound physiologically equivalent to them.

TABLE 7.—*Growth of H. parainfluenzae in the Presence of Past. suiseptica Filtrates*

Dextrose Veal Infusion Broth plus		Growth of H. parainfluenzae
Diphosphopyridine nucleotide (control)	(0.05μg. per cc.)	+++
Suiseptica filtrate	(0.1 cc.)	++
	(0.3 cc.)	+++
Heat treated suiseptica filtrate	(0.1 cc.)	++
	(0.3 cc.)	+++
Control		0

Use of synthetic medium.—Since a basal medium of hydrolyzed purified gelatin was used in most of the previous experiments, attempts were also made to grow the hemorrhagic septicemia pasteurellas in a basal medium of definite chemical composition. It was found that 16 of the 17 strains studied could be grown in a medium consisting of 18 amino acids, inorganic salts and glucose if nicotinamide, pantothenic acid and biotin concentrate were added. Growth under these conditions was, in general, not as rapid or luxuriant as when the hydrolyzed gelatin basal medium was used. Several strains which had given fairly good growth in the hydrolyzed gelatin medium now produced only a scanty growth. The results of such an experiment are shown in table 8.

A number of attempts to make successive transfers of cultures grown in the synthetic medium proved unsuccessful in the majority of cases. Usually after two or three such transfers the cultures failed to grow. This is in marked contrast to the results obtained in the hydrolyzed gelatin basal medium in which the organisms can apparently develop indefinitely in successive transfers.

Efforts to make these cultures develop more readily in the synthetic medium by the addition of other factors among which were vitamin K, choline, para-amino-benzoic acid and oleic acid also were unsuccessful. Evidently something in the hydrolyzed gelatin, whose nature we do not know, is needed by these organisms. This might be an accessory factor. On the other hand the poorer growth in synthetic medium may

TABLE 8.—*Growth of Hemorrhagic Septicemia Pasteurellas in Synthetic Medium with Certain Growth Factors*

Pasteurella	Synthetic Basal Medium plus Nicotinamide and Pantothenic Acid*			Synthetic Basal Medium plus Nicotinamide, Pantothenic Acid and Biotin Concentrate*	
	1	4	days	1	4
suiseptica S230	+++	+++		+++	+++
cuniculicida 7229	0	0		0	0
oviseptica 1	0	±		0	±
oviseptica 2	0	±		+	±
bubaliseptica 41	+	++		+	++
bubaliseptica MH718	0	0		+	±
bubaliseptica 888	+	++		+	+++
boviseptica 53	+	++		++	+++
boviseptica 221	0	0		+++	+++
boviseptica 590L	+	++		++	+++
boviseptica 18	0	0		±	+
suiseptica 100	+	+		++	+++
suiseptica DM2	+	++		++	+++
avicida 1525	+	+		++	+++
avicida NY81	+	++		+	++
avicida	0	+		+	+
aviseptica 351	+	++		++	++

The organisms did not develop in the synthetic basal medium alone.
* Nicotinamide and pantothenic acid were added in amounts of 0.1μg. per cc. each. Biotin concentrate was added in the amount of 0.15μg. per cc.

be due to the lack of some amino acid or to incorrect proportions of amino acids, to the lack of some inorganic salt, or to variation in the physical character of the medium.

PASTEURELLA TULARENSE

As seen in table 1, these organisms did not develop in the basal medium alone but growth appeared within about 4 days when the accessory mixture was added. On simplifying the accessory mixture it was found that either thiamin or cocarboxylase (diphosphothiamin) is essential for the development of these organisms. While the organisms multiplied slowly, the turbidity became marked within 4 to 7 days. When a very light inoculum was used in these experiments growth often failed to appear, but when the inoculum was increased (definite, though light turbidity in the salt solution tube from which the 0.1 cc. inoculations were made) growth appeared regularly when the proper accessory was added. This larger inoculum, however, was not sufficient to induce growth in the control tubes of basal medium which lacked the accessory factors. Results are shown in table 9.

In contrast to the results in the hydrolyzed gelatin medium these organisms did not develop at all in the amino acid basal medium when the accessory factors were added.

The tularemia organisms have been reported at various times to need blood and cystine for regular development, but the results of the present work indicate that Past. tularense can develop without blood. The addition of hemin did not have a stimulating effect on growth. If cystine is needed, then the amount present in the basal medium of hydrolyzed gelatin (20 mg. per liter) was sufficient, since the addition of more cystine did not aid in the development of these strains.

In an attempt to discover whether an environment rich in oxygen would act as an additional stimulus to growth of these organisms an experiment was conducted in which they were inoculated into tubes with and without accessory factors and grown in atmospheres of 10, 25 and 50% of a mixture of 95% oxygen and 5% carbon dioxide. They showed no better development under these conditions than in the usual tests.

Since the slow growth in the presence of thiamin occurred regularly on repeated tests, and since growth did not occur in the absence of thiamin, it was concluded that thiamin is one of several

TABLE 9.—*Growth of Past. tularense in Hydrolyzed Gelatin Basal Medium and with Added Accessory Factors*

Past. tularense Strains	Hydrolyzed Gelatin Basal Medium plus Accessory Mixture			Thiamin*			Cocarboxylase*			Control		
	days											
	1	4	7	1	4	7	1	4	7	1	4	7
395	0	++	+++	0	++	+++	0	++	+++	0	0	0
399	0	++	+++	0	++	+++	0	++	+++	0	0	0
405	0	++	+++	0	++	+++	0	++	+++	0	0	0
408	0	++	+++	0	++	+++	0	++	+++	0	0	0
410	0	++	+++	0	++	+++	0	++	+++	0	0	0
413	0	++	+++	0	0	+++	0	++	+++	0	0	0
421	0	++	+++	0	0	++	0	++	++	0	0	0
424	0	++	+++	0	0	+++	0	+	+++	0	0	0
425	0	+++	+++	0	+++	+++	0	+++	+++	0	0	0
426	0	+++	+++	0	+++	+++	0	+++	+++	0	0	0
Tenn.	0	++	+++	0	+	+++	0	+	+++	0	0	0
407	0	++	+++	0	++	+++	0	++	+++	0	0	0
396	0	++	+++	0	++	+++	0	++	+++	0	0	0
Pack	0	++	+++	0	+	+++	0	+	+++	0	0	0
38	0	0	0	0	0	0	0	0	0	0	0	0

* Thiamin and cocarboxylase were added in amounts of 0.1μg. per cc. each.

factors needed for development of these organisms; the other factors are not known.

PASTEURELLA PESTIS AND PASTEURELLA PSEUDOTUBERCULOSIS

As shown in table 1, 9 of the 10 strains of these species grew quite readily in the hydrolyzed gelatin basal medium and addition of the accessory mixture did not improve growth in any instance. It was further found that these organisms grew about as well in the amino acid synthetic basal medium as in the basal medium of hydrolyzed gelatin. Rao[9] recently reported that Past. pestis will develop in a medium of amino acids, inorganic salts and glucose and his findings are confirmed here for 4 of the 5 strains tested.

In one instance, Past. pestis 122, growth did not occur in the synthetic basal medium alone or when the accessory factors were added, while slight growth did appear after 4 days in the hydrolyzed gelatin basal medium (table 1). In this one case then, the hydrolyzed gelatin seemed to have a factor or factors, absent from the synthetic basal medium, which were necessary for this particular strain.

DISCUSSION

It is interesting to consider the results of this study in relation to classification of the group. The genus Pasteurella is often separated into 4 general subdivisions, 2 of which, Past. pestis and Past. pseudotuberculosis have been shown to have a definite immunological relationship to each other.[10] If, then, we assume that the genus actually has 3 unrelated subgroups we find that the nutritional requirements of these 3 subdivisions vary quite widely. On the one extreme there are Past. pestis and Past. pseudotuberculosis which for the most part require none of the known accessory growth factors and which develop readily in a medium of amino acids, inorganic salts and glucose. Then there is a subgroup, the hemorrhagic septicemia pasteurellas, which can develop readily on ordinary laboratory mediums but which cannot develop in hydrolyzed gelatin or in amino acid mediums unless nicotinamide and pantothenic acid are added. On the other extreme there is the Past. tularense group which will develop only on enriched laboratory mediums, slowly in hydrolyzed gelatin medium in the presence of thiamin and relatively large inoculum of cells, and not at all in an amino acid medium enriched with the accessory growth factors known at the present time.

The 3 unrelated subgroups included in this genus produce distinctly recognizable types of disease in man or in man and animals. They also show different physiological and biochemical characteristics and, from the results reported here, distinctly different accessory growth factor requirements. These facts would seem to indicate a correct and solid fundamental basis for the subgroups within the genus.

SUMMARY

A study of the accessory growth factor requirements of the genus Pasteurella may be summarized as follows:

The hemorrhagic septicemia pasteurellas developed in a hydrolyzed gelatin basal medium when nicotinamide (or di- or triphosphopyridine nucleotide) and pantothenic acid were added. These organisms also grew in an amino acid medium in the presence of these factors through 2 or 3 transfers but not through repeated transfers.

Biotin concentrate was shown to contain some substance needed by many

9. Indian J. M. Research **27**: 75, 1939.
10. MacConkey, A. T.: J. Hyg. **8**: 335, 1908.

hemorrhagic septicemia pasteurellas but pure biotin could not replace this unknown factor.

It was found that nicotinic acid could not substitute for nicotinamide or diphosphopyridine nucleotide in supporting growth.

Methylene blue reduction and oxygen uptake with washed cell suspensions of the hemorrhagic septicemia pasteurellas showed marked stimulation in the presence of diphosphopyridine nucleotide and nicotinamide, but no stimulation in the presence of nicotinic acid.

A number of other compounds closely related to nicotinic acid, which have been reported active for other organisms requiring nicotinic acid or amide, did not support growth of the hemorrhagic septicemia pasteurellas.

These organisms converted nicotinamide to diphosphopyridine nucleotide or to some substance physiologically similar to it.

The component parts of pantothenic acid, alone or in combination, did not substitute for the intact molecule.

Past. tularense grew slowly in a hydrolyzed gelatin basal medium plus thiamin or cocarboxylase. These cultures did not develop in an amino acid medium with the addition of known accessory growth factors.

Four of 5 strains of Past. pestis, 5 of Past. pseudotuberculosis and the 1 strain of Past. hemolytica developed readily in an amino acid medium in the absence of accessory growth factors.